# GENE MUTATION ASSOCIATED WITH AGE-RELATED MACULAR DEGENERATION

## CROSS REFERENCE TO RELATED APPLICATION

This Application claims the benefit of Provisional U.S. Patent Application No. 60/443,214 , filed January 27, 2003.

## FIELD OF THE INVENTION

The present invention relates generally to the field of age-related macular degeneration (AMD). More particularly, the invention is concerned with the identification of a gene which, when mutated, is associated with AMD, as well as the transcripts, gene products and associated sequence information. The present invention also relates to methods for diagnosing and detecting carriers of the gene, and to AMD diagnosis and gene therapy using the information derived from the DNA, protein, and function of the protein.

## BACKGROUND OF THE INVENTION

AMD involves a loss of central vision as a result of a progressive degeneration of retinal and underlying tissues in people over the age of 50 years. Specific causes of significant loss in AMD are atrophy of the retina and the retinal pigment epithelium, and/or the growth of neovascularization with subsequent subretinal scarring. It is the main cause of irreversible blindness in the United States and Europe, and the prevalence appears to be increasing. Increasing rates are also being documented in Asia (Koh, A.H.C., et al., Ann. Acad. Med. 31:399-404, 2002). AMD accounts for about 50% of all cases of registered blindness in the west. Approximately 2 million Americans suffer vision loss from AMD, and over 10 million Americans show some early signs of AMD. The incidence of significant vision loss associated with AMD is about 2% for those at age 70, and 6% for those at age 80. (Hawkins, B.S., *et al.*, Mol. Vis. 5:26, 1999; Vingerling, J. R., *et al.* Epidemiol. Rev. 17:347-360, 1995.) The total number of people with AMD is expected to triple by the year 2030. (Vinding, T., Acta Ophthalmologica 73 (Suppl):1-32, 1995.)

Current methods of treatment have achieved only limited efficacy, and are ineffective if not started at a relatively early stage of the disease. Thus, there is a critical need in the art for

methods of detecting AMD at a stage early enough to permit therapeutic treatment to prevent loss of vision, and to ultimately prevent development of the disease.

BRIEF DESCRIPTION OF THE FIGURES

**Figure 1.** Fundus photographs of representative affected members of family A. **Panel A**. Right eye of 54-year old individual (III-13) with extensive large drusen. **Panel B.** Left eye of 87-year old individual (II-9) with geographic atrophy and drusen.

**Figure 2.** Depiction of the domain structure of FIBL-6 and conservation of Gln5345. The amino acid sequence of a portion of exon 104 in human, 7 additional mammalian species, and chicken is provided.

**Figure 3.** Chromatograms of fluorescent dye-terminator sequencing of PCR products of FIBL-6 demonstrating an A→G change (boxed) at the second nucleotide of codon 5345, which is expected to produce the Gln5345Arg change. Heterozygote: both chromosomes of the proband (III-3) of family A. Wildtype: paternal chromosome of III-3. Mutant: maternal chromosome of III-3.

**Figure 4.** Family A pedigree showing that the Gln5345Arg variation segregates exclusively with the disease haplotype. Disease haplotype and Gln5345Arg variation are boxed. Squares denote males; circles, females; slashed symbol, deceased; filled, affected; N, normal; ?, uncertain; open, undiagnosed; quarter-filled, suspected affected; and arrow, proband. The smaller numbers above the gender symbols indicate age at death or diagnosis. The marker D1S2127 is about 965 kb proximal to *FIBL-6*, D1S254 and D1S191 are within *FIBL-6*, and D1S202 is about 806 kb distal to *FIBL-6*.

**Figure 5.** Evidence of a common founder. Pedigrees of families B, C, and D show an association of the Gln5345Arg variation with a unique haplotype also found in family A. Family A disease haplotype and genotypes of the sporadic case and two control subjects with the variation are displayed at the bottom. The unique haplotype and Gln5345Arg variation are boxed (dark shading). Genotypes that cannot be assigned to haplotypes are boxed (light shading). Squares denote males; circles, females; slashed symbol, deceased; filled, affected; N, normal; ?, uncertain; open, undiagnosed; quarter-filled, suspected affected; and arrow, proband.

The smaller numbers above the gender symbols indicate age at death or diagnosis. The most proximal shared allele, from marker D1S240, is not shown for clarity. D1S2711 is about 186 kb proximal to *FIBL-6*, D1S444, D1S254, D1S191, and D1S2848 are within *FIBL-6*, and D1S2138 is about 206 kb distal to *FIBL-6*.

**Figure 6.** FIBL-6 expression and alternative splicing of exon 104. **A**. Upper two sets of bars represent PCR amplicons obtained with 5' primer in exon 103 and 3' primer in exon 105 (left-hand panels in B and C; 185 bp product indicates absence of exon 104); lower bar represents amplicon obtained with identical 5' primer but with 3' primer in exon 104 (right-hand panels in B and C). **B**. Expression in RPE cells and skin fibroblasts. **C**. Expression in iris, retina, and choroid tissue.

**Figure 7**. Figure 7 shows the polynucleotide sequences of human fibulin 6 mRNA, accession no. XM_053531.6, also as shown in SEQ ID NO:1.

**Figure 8**. Figure 8 shows the amino acid sequence of human fibulin 6, also shown as SEQ ID NO:2.

## DETAILED DESCRIPTION OF THE INVENTION

Age-related macular degeneration (AMD) is a major cause of blindness in the United States and other industrialized nations. (Evans J, Wormald R., *British Journal Ophthalmology* 80:9-14, 1996; Klein R, Klein BEK, Linton KLP, *Ophthalmology* 99:933-943, 1992; Vingerling JR, *Ophthalmology* 102:205-210, 1995). Early AMD is characterized clinically by drusen, which are extracellular deposits of proteins, lipids, and cellular debris, (Hageman GS, Mullins RF, *Mol Vis* 5:28, 1999), that are located beneath the retinal pigment epithelium (RPE). The RPE provides nutritional, metabolic, and phagocytic functions for the overlying photoreceptors. Significant vision loss results from dysfunction or death of photoreceptors in the macula in association with late stages of AMD (geographic atrophy of the RPE and subretinal neovascularization). The late onset of AMD and its apparent complex genetics have hindered mapping and identification of genes that cause this disease. (Gorin MB, *Mol Vis* 5:29, 1999.)

Although several forms of therapy are available, most patients with AMD continue to lose vision in spite of treatment. Laser photocoagulation has been used for over three decades, but is of limited value in only a comparatively small proportion of cases. In the past three years, photodynamic therapy has been used to treat a somewhat larger proportion of AMD cases. However, the benefits are limited primarily to slowing the progression of the disease. Currently, pharmacologic therapy is being actively investigated. Several anti-angiogenic drugs are being evaluated in ongoing clinical trials, with no definitive results at this time. Radiation therapy has not been shown to be of benefit, and a number of surgical approaches are also under investigation, but with no conclusive benefits shown.

Because of the lack of suitable and successful treatment options, early diagnosis and prevention are goals in current AMD research. One recently completed large study demonstrated that treatment of certain patients with "high risk" AMD using antioxidants and zinc led to a statistically significant reduction in the occurrence of moderate visual loss. Disease progression was also reduced by approximately 25% in these patients. Thus, there is some indication that non-surgical intervention can be helpful in at least slowing the progression of the disease.

It is preferable to slow or cease the progress of the disease at a stage where the patient has minimal loss of vision. This involves the need for methods of early detection, so that a treatment regimen, such as anti-oxidant therapy, can be initiated. While it had been believed that AMD was an acquired disease, more recent evidence shows a clear hereditary element in at least some forms of the disease. In these involved families, as treatment regimens improve, early intervention will be very important.

A mutation in EGF-containing fibrillin-like extracellular matrix protein 1 (EFEMP1) was identified and reported by Stone, E.M., *et al.*, Nature Genetics 22:199-202, 1999. The mutation is associated with Malattia Leventinese (ML) and Doyne honeycomb retinal dystrophy (DHRD), both of which are autosomal dominant diseases characterized by drusen deposits that accumulate beneath the retinal pigment epithelium. Stone *et al.* identified a single non-conservative mutation, in which arginine is replaced by tryptophan at position 345. The mutation was found in all families studied who exhibited the ML/DHRD phenotype. The mutation was not found in 477 control individuals or in 494 patients with age-related macular degeneration. This research in

part set a precedent for the proposition that a single mutation could be closely associated with phenotype in a disease with clinical symptoms similar to those of macular degeneration, particularly the accumulation of drusen. Another useful clue from this work was the discovery of the mutation.

The present inventors previously identified a large family affected by AMD, and found linkage of the defect to chromosome 1q. Klein, M. L., *et al.*, "Age-Related Macular Degeneration. Clinical Features in a Large Family and Linkage to Chromosome 1q," Arch. Ophthalmol. 116:1082-1088, 1998. In this large family, transmission of AMD trait is consistent with autosomal-dominant inheritance. The average age at diagnosis of AMD was 65 years, and the earliest age of onset of symptoms was 52 years. In two family members, the disease haplotype was found but the individuals did not exhibit the phenotype, possibly because of their ages, 44 and 48, which were below the earliest age of onset for the individuals exhibiting the disease. Diagnosis was performed using the established Wisconsin Age-Related Maculopathy Grading System, as described in Klein, R., *et al.*, Ophthalmology 104:7-21, 1997.

These early studies of the family led to the conclusion that the disease-causing gene for AMD in this family was located between markers D1S466 and D1S413 (Figure 4). Klein ML, *Arch Ophthalmol* 116:1082-8, 1998. Identification of this region led the inventors to consider the possibility of a mutation as a causative factor in the inherited form of AMD in this large family. The inventors screened 27 genes from this region for sequence variations that segregate with the disease haplotype in this family.

According to the invention, *FIBULIN-6* (*FIBL-6*) was screened because of its resemblance to *EFEMP1*. The protein sequence of FIBL-6 is similar to that of previously identified hemicentin in *Caenorhabditis elegans*. (Carpten JD, *Genomics* 64:1-14, 2000; Vogel BE, Hedgecock EM. *Development* 128:883-94, 2001; Hubbard T, *Nucleic Acids Res* 30:38-41, 2002.) *FIBL-6* maps to 1q25.3-1q31.1, and extends over 450 kb of genomic DNA (http://www.Ensembl.org/Homo_sapiens/geneview?gene=ENSG00000143341). Pairwise alignment (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) of the FIBL-6 mRNA with genomic sequence delineated 107 exons that encode a 5,635 amino acid protein with a calculated molecular weight over 600 kDa. The predicted protein consists of an N-terminal von Willebrand

factor type A domain, 44 tandem immunoglobulin modules, 6 thrombospondin type 1 domains, a G2 nidogen domain, 7 calcium binding epidermal growth factor-like (cbEGF) domains, and 1 EGF-like domain (Figure 2).

*FIBL-6* was considered to be an excellent candidate for the *ARMD1* gene because its encoded protein contains a series of predicted cbEGF domains followed by a single EGF-like domain at its carboxy terminus similar to EFEMP1 (http://smart.embl-heidelberg.de/smart). This final EGF-like domain of EFEMP1 harbors the mutation associated with Mallatia Leventinese, Stone EM, *Nat Genet* 22:199-202, 1999, an earlier onset macular dystrophy characterized by drusen similar to those seen with AMD.

As a result of these analyses, a sequence variation was identified in exon 104 of the FIBL-6 gene, which produces a change from Gln to Arg at position 5345. This variation segregated with the disease haplotype in the family discussed above that had been linked to 1q25-31. This change was also detected in some affected members of three other AMD families, two unaffected members, one sporadic AMD case, and two control subjects. Individuals with the variation may appear normal due to their relatively young age or other phenotypic determinants. Gln5345 of FIBL-6 is conserved among eight species analyzed. RT-PCR analysis demonstrated FIBL-6 mRNA in human skin fibroblasts and retinal pigment epithelium (RPE) cells, as well as porcine trabecular meshwork cells, sclera, and retina. Two transcripts were identified, one with exon 104 and one without. The evidence of segregation, similarity to *EFEMP1* and protein conservation, taken together, support the conclusion that the Gln5345Arg variation in FIBL-6 is the mutation responsible for AMD in this large family, which maps to 1q25-31.

Stone *et al.* reported that a single non-conservative mutation, Arg345Trp, in the gene *EFEMP1* was identified in families of individuals afflicted with Malattia Leventinese and Doyne honeycomb retinal dystrophy. These diseases appear earlier than does AMD. *EFEMP1* refers to EGF-containing fibrillin-like extracellular matrix protein 1. (Nature Genetics 22:199-202, 1999.) The mutation identified in the present AMD family affects a protein of the FIBL-6 family. A human ortholog of hemicentin, which had been studied in nematodes (Vogel, B. E., *et al.*, Development 128:883-894, 2001), was identified in chromosome 1q24-25 (Carpten, J. D., *et*

*al.*, Genomics 64:1014, 2000). FIBL-6 is an extracellular protein that, studies suggest, plays a role in ordering epithelial cells and in intracellular connections. Vogel *et al.* found that mutations in FIBL-4 in nematodes is associated with a syndrome of tissue fragility, defective cell migration, among other effects.

Overall, the disclosed evidence provides strong support that *FIBL-6* is the *ARMD1* gene. The Gln5345Arg variation segregates exclusively with the *ARMD1* disease haplotype in the 27-member family A. Of the 146 variations in 27 genes screened by the inventors to date, Gln5345Arg is the only variation with this property. No other changes in *FIBL-6* were detected in the proband from family A. The Gln5345Arg variation appears to have a common founder among all four families and the three separate individuals studied. The glutamine at position 5345 in human FIBL-6 is strictly conserved among the 8 species analyzed. The glutamine to arginine variation changes both the size and the charge of the amino acid side chain, thus protein structure and function are likely to be affected.

*FIBL-6* was a likely candidate for the *ARMD1* gene because of its extensive similarity to *EFEMP1*. Both of these fibulin family genes encode an extracellular matrix protein that has a series of cbEGF domains followed by a single EGF-like domain at the carboxy terminus. Also, analogous to the Arg345Trp mutation in *EFEMP1*, which causes Mallatia Leventinese and Doyne honeycomb retinal dystrophy, there may be only a single *FIBL-6* variant associated with AMD that has been inherited from a common founder. However, in contrast to *EFEMP1*, there may be additional environmental and genetic factors that determine the phenotypic expression of AMD.

In family A, only family members with the disease haplotype carry the Gln5345Arg mutation. One family member (III-18), who carries neither the disease haplotype nor the sequence variation, was diagnosed with AMD. However, both her mother (II-9), who is a family member, and her father (II-10) were diagnosed with AMD. Since AMD is considered a prevalent, Klein R, Klein BEK, Linton KLP, *Ophthalmology* 99:933-943, 1992, and complex heterogeneous disorder, Gorin MB, *Mol Vis* 5:29, 1999; Evans K, Bird AC, *British Journal of Ophthalmology* 80:763-768, 1996; Stone EM, Sheffield VC, Hageman GS, *Hum Mol Genet* 10:2285-92, 2001; Yates JR, Moore AT, *J Med Genet* 37:83-87, 2000, the father (II-10) and

daughter's (III-18) AMD may be due to another gene. Of the four remaining family members who were not diagnosed with AMD but carry the Gln5345Arg change, one was not available for examination, and three, who were under 50 years of age, were likely too young to exhibit AMD.

The Gln5345Arg variation was also found in three additional AMD families, in which the variation does not segregate with AMD, and two control subjects. However, all deviations from a Mendelian pattern of inheritance can be explained by complex genetics, which are thought to be associated with AMD (Klein R, Klein BEK, Linton KLP, *Ophthalmology* 99:933-943, 1992), and complex heterogeneous disorder (Gorin MB, *Mol Vis* 5:29, 1999; Evans K, Bird AC, *British Journal of Ophthalmology* 80:763-768, 1996; Stone EM, Sheffield VC, Hageman GS, *Hum Mol Genet* 10:2285-92, 2001; Yates JR, Moore AT, *J Med Genet* 37:83-87, 2000). In particular, in all three nuclear families where the variation is found, both parents may carry genes that lead to AMD. Consistent with this notion, the frequency of AMD in these nuclear families is higher than 50% (i.e., 9 of 11 children ascertained have AMD). The two control subjects and the one unaffected member of family B may be either too young to exhibit signs of AMD, contain compensating changes within FIBL-6, lack other phenotypic determinants for AMD, or harbor genes that protect them from AMD.

In this study, no evidence was found to demonstrate that the Gln5345Arg variation has occurred on different chromosomal backgrounds. Rather, it appears to have a common founder. In all four families studied and probably in the two control subjects and lone sporadic case, the Gln5345Arg variation is associated with a unique shared haplotype. In families B and C, this haplotype could not have originated from the parent who is the family member (Figure 5). In family D, the other affected family members do not share this unique haplotype.

In order to confirm the inheritance of the Gln5345Arg variation from the spouse (II-1) in family C, DNA has been obtained from some of his relatives, including a niece and a nephew. Although the 85-year old nephew, whose diagnosis is uncertain for AMD (Klein ML, *Arch Ophthalmol* 116:1082-8, 1998), does not carry the Gln5345Arg variation, the 89-year old niece, who is not affected, does carry the variation. She also shares the same unique haplotype that is found associated with the variation as well as a more extensive haplotype that is found in the two affected family C members (III-2 and III-3) that carry the variation. This demonstrates that the

spouse (II-1) was the source of the variation in family C. This also suggests the possibility of a protective gene for AMD or that other genetic and environmental factors may contribute to AMD.

The degree of conservation of an amino acid across species is often an indication of its functional importance. (Valdar WS, *Proteins* 48:227-41, 2002.) Gln5345 is conserved in seven other mammals and chicken, suggesting an important functional role. In most cbEGF domains, this position is occupied by either tyrosine or phenylalanine. It is usually involved in a hydrophobic packing interaction with a glycine in the adjacent downstream cbEGF domain. (Handford PA., *Biochim Biophys Acta* 1498:84-90, 2000.) However, in FIBL-6 it is conserved as glutamine in the novel cbEGF domain encoded by exon 104. This domain is novel because it contains an additional 76 amino acids at its carboxy terminus instead of the usual one or two that link tandem cbEGF domains (Downing AK, Knott V, *Cell* 85:597-605, 1996), and because exon 104 exhibits alternative splicing. In general, FIBL-6 appears to be a very highly conserved protein. The overall amino acid identity between the mouse and human FIBL-6 sequence is 4745/5751 or 82%. In twelve unique human copies of FIBL-6, there are only seven non-synonymous changes besides the Gln4345Arg variation.

The ortholog of FIBL-6, hemicentin in *C. elegans*, is associated with a variety of cell-matrix and cell-cell interactions. (Vogel BE, *Development* 128:883-94, 2001.) It functions between neurons and epidermis but elsewhere functions across basement membranes between tissues. *FIBL-6* may thus be involved in interactions where drusen form, between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane. Interestingly, other members of the fibulin gene family are located within chromosomal regions recently identified in genome-wide scans for AMD genes (Iyengar et al., *ARVO*; 2002:Abstract #1844.). The fibulin family of proteins may play a major role in AMD and other macular dystrophies.

The evidence is strong for a conclusion that the Gln5345Arg change in the FIBL-6 gene is responsible for AMD in a large family, and may contribute to the disease in three others. The appreciation of *FIBL-6* as the gene for *ARMD1*, along with the discovery of other genes that cause AMD, will result in earlier diagnosis of individuals potentially at risk for AMD, allow more appropriate screening to detect early disease at the stage where treatment might be most

efficacious, facilitate the investigation of environmental and genetic modifiers of the age of onset and severity of the phenotype, and allow sufficient understanding at the molecular and biochemical level such that rational gene-based therapies can be devised and tested. Modifier genes have already been reported to significantly affect the age of onset and expression of genetic disease. Examples of modifier genes include the Alu polymorphism in the angiotensin-converting enzyme (ACE) gene (Hamdi HK, *Biochem Biophys Res Commun* 295:668-72, 2002), and the null allele for ciliary neurotrophic factor (CNTF), which has a detrimental effect on the expression of amyotrophic lateral sclerosis from mutation of the SOD-1 gene. (Giess R, *Am J Hum Genet* 70:1277-86, 2002.) The study of the interaction of mutations of genes that cause AMD with sequence changes in other modifier genes will give new targets for pharmaceutical companies to create drugs that might prevent AMD or forestall the occurrence of the major vision loss in later stages.

The present invention is the first indication that a mutation in FIBL-6 in humans is associated with AMD. As such it provides the opportunity to detect the disease at an early stage, and to tailor therapy appropriately to affected family members, before the disease progresses to a point at which vision is irreversibly impaired. The identification of this mutation is also useful for providing methods and materials for elucidating the role of FIBL-6 in both normal retinal function, and loss of that function in AMD. Evidence that the glutamine affected by the mutation plays an important role in the function of FIBL-6 is indicated by the conservation of glutamine at that position in every mammalian species studied to date (mouse, rat, cat, dog, sheep, rabbit and pig), as well as chicken.

Methods are known in the art for identifying and screening for a polynucleotide sequence carrying a point mutation, and these methods are applicable to identifying carriers of the mutation of the present invention. U.S. Patent Publication 20010016323 discloses methods for detecting point mutations using a fluorescently labeled oligomeric probe and fluorescence resonance energy transfer. A point mutation leading to a base mismatch between the probe and the target DNA strand causes the melting temperature of the complex to be lower than the melting temperature for the probe and the target if the probe and target were perfectly matched. A suitable probe consists of SEQ ID NO:7, wherein this probe, 5'ACCAGGACAACATTTAT3', is a perfect match to the comparable region of the wild type gene. Another suitable probe is SEQ

ID NO:8, 5'ACCAGGACGACATTTAT3', which differs from SEQ ID NO:7 by a substitution of G for A. This probe is a perfect match for the corresponding region of the mutated gene of the invention. Thus, the invention provides probes for detecting the wild type gene by perfect match, and for detecting the mutated gene by perfect match. Other suitable probes comprise SEQ ID NO:7 or 8, in which one or more additional nucleotides are present at one of both ends of the probe, wherein the additional nucleotides do not interfere with the binding efficiency that is characteristic of the corresponding probe of SEQ ID NO:7 or 8. Examples of such probes include SEQ ID NO:12-94 for the wild-type gene, and SEQ ID NO:95-177 for the mutation.

Other suitable methods for detecting single point mutations include those disclosed in, for example, U.S. Patent Publication 2002010665, which involves the use of oligonucleotide probes in array format. Such arrays can include one or more of SEQ ID NO:7, 8, and 12-177. U.S. Patent Publication 20020177157 discloses additional methods for detecting point mutations.

A polynucleotide carrying the point mutation that is the subject of this invention can be identified using one or more of a number of available techniques. However, detection is not limited to the techniques described herein and the methods and compositions of the invention are not limited to these methods, which are provided for exemplary purposes only. Polynucleotide and oligonucleotide probes are also disclosed herein and are within the scope of the invention, and these probes are suitable for one or more of the techniques described below. These include allele-specific oligo hybridization (ASO), which, in one embodiment, is a diagnostic mutation detection method wherein hybridization with a pair of oligos corresponding to alleles of a known mutation is used to detect the mutation. Another suitable method is denaturing high performance liquid chromatography (DHPLC), which is a liquid chromatography method designed to identify mutations and polymorphisms based on detection of heteroduplex formation between mismatched nucleotides. Under specified conditions, heteroduplexes elute from the column earlier than homoduplexes because of reduced melting temperature. Analysis can then be performed on individual samples.

An amplified region of the DNA containing the mutation or the wild-type sequence can be analyzed by DHPLC. In the present invention, DHPLC was performed on a 194 base pair PCR product generated using the forward primer SEQ ID NO:3 and the reverse primer SEQ ID NO:4. Use of DHPLC is described in U.S. Patent Nos. 5,795,976 and 6,453,244, both of which

are incorporated herein by reference. A suitable method is that provided by Transgenomic, Inc. (Omaha, NE) using the Transgenomic WAVE® System.

For ASO, a region of genomic DNA or cDNA containing the mutation is amplified by PCR and transferred onto duplicating membranes. This can be performed by dot/slot blotting, spotting by hand, or digestion and Southern blotting. The membranes are prehybridized, then hybridized with a radiolabelled or deoxygenin (DIG) labelledoligonucleotide to either the mutant or wild-type sequences. For the DIG label, detection is performed using chemiluminesccent or colorimetric methods. The membranes are then washed with increasing stringency until the ASO is washed from the non-specific sequence. Following autoradiographic exposure, the products are scored for the level of hybridization to each oligo. Optimally, controls are included for the normal and mutant sequence on each filter to confirm correct stringency, and a negative PCR control is used to check for contamination in the PCR.

The size of the ASO probe is not limited except by technical parameters of the art. Generally, too short a probe will not be unique to the location, and too long a probe may cause loss of sensitivity. The oligos are preferably 15-21 nucleotides in length, with the mismatch towards the center of the oligo. Most preferable oligos for the present invention include SEQ ID NO:7 for the wild-type, and SEQ ID NO:8 for the mutant sequence. These oligos represent nucleotides of position 16,255 to position 16,271 of SEQ ID NO:1. For SEQ ID NO:8, position 16,263 is G instead of A. Other suitable wild-type oligos include nucleotides of position 16,256 to position 16,272; 16,257 to 16,273; 16,258 to 16,274; and 16,259 to 16,275, all with reference to SEQ ID NO:1. For the corresponding mutant oligo, in each case, position 16,263 is G instead of A. The foregoing examples are 17-mer oligos. Also suitable are 15, 16, 18, 19, 20, and 21-mer oligos, wherein each of the oligos described here can be extended at one or both ends by one or more nucleotides as shown in the corresponding region of SEQ ID NO:1, and in each case the wild-type and mutant oligos differ at position 16,263, which is A for the wild-type oligo and G for the mutant oligo.

Exemplary wild-type oligos, with the "A" at position 16,263 of SEQ ID NO:1 indicated in bold, include:

<u>15-mers</u>:

GAC**A**ACATTTATTAG (SEQ ID NO:12)

GGAC**A**ACATTTATTA (SEQ ID NO:13)

AGGACAACATTTATT (SEQ ID NO:14)
CAGGACAACATTTAT (SEQ ID NO:15)
CCAGGACAACATTTA (SEQ ID NO:16)
ACCAGGACAACATTT (SEQ ID NO:17)
CACCAGGACAACATT (SEQ ID NO:18)
CCACCAGGACAACAT (SEQ ID NO:19)
TCCACCAGGACAACA (SEQ ID NO:20)

16-mers:

GACAACATTTATTAGG (SEQ ID NO:21)
GGACAACATTTATTAG (SEQ ID NO:22)
AGGACAACATTTATTA (SEQ ID NO:23)
CAGGACAACATTTATT (SEQ ID NO:24)
CCAGGACAACATTTAT (SEQ ID NO:25)
ACCAGGACAACATTTA (SEQ ID NO:26)
CACCAGGACAACATTT (SEQ ID NO:27)
CCACCAGGACAACATT (SEQ ID NO:28)
TCCACCAGGACAACAT (SEQ ID NO:29)
GTCCACCAGGACAACA (SEQ ID NO:30)

17-mers:

GACAACATTTATTAGGG (SEQ ID NO:31)
GGACAACATTTATTAGG (SEQ ID NO:32)
AGGACAACATTTATTAG (SEQ ID NO:33)
CAGGACAACATTTATTA (SEQ ID NO:34)
CCAGGACAACATTTATT (SEQ ID NO:35)
ACCAGGACAACATTTAT (SEQ ID NO:7)
CACCAGGACAACATTTA (SEQ ID NO:36)
CCACCAGGACAACATTT (SEQ ID NO:37)
TCCACCAGGACAACATT (SEQ ID NO:38)
GTCCACCAGGACAACAT (SEQ ID NO:39)

TGTCCACCAGGACAACA (SEQ ID NO:40)

18-mers:

| | |
|---|---|
| GACAACATTTATTAGGGG | (SEQ ID NO:41) |
| GGACAACATTTATTAGGG | (SEQ ID NO:42) |
| AGGACAACATTTATTAGG | (SEQ ID NO:43) |
| CAGGACAACATTTATTAG | (SEQ ID NO:44) |
| CCAGGACAACATTTATTA | (SEQ ID NO:45) |
| ACCAGGACAACATTTATT | (SEQ ID NO:46) |
| CACCAGGACAACATTTAT | (SEQ ID NO:47) |
| CCACCAGGACAACATTTA | (SEQ ID NO:48) |
| TCCACCAGGACAACATTT | (SEQ ID NO:49) |
| GTCCACCAGGACAACATT | (SEQ ID NO:50) |
| TGTCCACCAGGACAACAT | (SEQ ID NO:51) |
| CTGTCCACCAGGACAACA | (SEQ ID NO:52) |

19-mers:

| | |
|---|---|
| GACAACATTTATTAGGGGA | (SEQ ID NO:53) |
| GGACAACATTTATTAGGGG | (SEQ ID NO:54) |
| AGGACAACATTTATTAGGG | (SEQ ID NO:55) |
| CAGGACAACATTTATTAGG | (SEQ ID NO:56) |
| CCAGGACAACATTTATTAG | (SEQ ID NO:57) |
| ACCAGGACAACATTTATTA | (SEQ ID NO:58) |
| CACCAGGACAACATTTATT | (SEQ ID NO:59) |
| CCACCAGGACAACATTTAT | (SEQ ID NO:60) |
| TCCACCAGGACAACATTTA | (SEQ ID NO:61) |
| GTCCACCAGGACAACATTT | (SEQ ID NO:62) |
| TGTCCACCAGGACAACATT | (SEQ ID NO:63) |
| CTGTCCACCAGGACAACAT | (SEQ ID NO:64) |
| TCTGTCCACCAGGACAACA | (SEQ ID NO:65) |

20-mers:

GACAACATTTATTAGGGGAC (SEQ ID NO:66)
GGACAACATTTATTAGGGGA (SEQ ID NO:67)
AGGACAACATTTATTAGGGG (SEQ ID NO:68)
CAGGACAACATTTATTAGGG (SEQ ID NO:69)
CCAGGACAACATTTATTAGG (SEQ ID NO:70)
ACCAGGACAACATTTATTAG (SEQ ID NO:71)
CACCAGGACAACATTTATTA (SEQ ID NO:72)
CCACCAGGACAACATTTATT (SEQ ID NO:73)
TCCACCAGGACAACATTTAT (SEQ ID NO:74)
GTCCACCAGGACAACATTTA (SEQ ID NO:75)
TGTCCACCAGGACAACATTT (SEQ ID NO:76)
CTGTCCACCAGGACAACATT (SEQ ID NO:77)
TCTGTCCACCAGGACAACAT (SEQ ID NO:78)
ATCTGTCCACCAGGACAACA (SEQ ID NO:79)

21-mers:

GACAACATTTATTAGGGGACG (SEQ ID NO:80)
GGACAACATTTATTAGGGGAC (SEQ ID NO:81)
AGGACAACATTTATTAGGGGA (SEQ ID NO:82)
CAGGACAACATTTATTAGGGG (SEQ ID NO:83)
CCAGGACAACATTTATTAGGG (SEQ ID NO:84)
ACCAGGACAACATTTATTAGG (SEQ ID NO:85)
CACCAGGACAACATTTATTAG (SEQ ID NO:86)
CCACCAGGACAACATTTATTA (SEQ ID NO:87)
TCCACCAGGACAACATTTATT (SEQ ID NO:88)
GTCCACCAGGACAACATTTAT (SEQ ID NO:89)
TGTCCACCAGGACAACATTTA (SEQ ID NO:90)
CTGTCCACCAGGACAACATTT (SEQ ID NO:91)
TCTGTCCACCAGGACAACATT (SEQ ID NO:92)
ATCTGTCCACCAGGACAACAT (SEQ ID NO:93)
TATCTGTCCACCAGGACAACA (SEQ ID NO:94)

Exemplary oligos for detecting the mutation, with a "G" instead of an "A" at position 16,263 of SEQ ID NO:1 indicated in bold, include:

15-mers:

GAC**G**ACATTTATTAG (SEQ ID NO:95)
GGAC**G**ACATTTATTA (SEQ ID NO:96)
AGGAC**G**ACATTTATT (SEQ ID NO:97)
CAGGAC**G**ACATTTAT (SEQ ID NO:98)
CCAGGAC**G**ACATTTA (SEQ ID NO:99)
ACCAGGAC**G**ACATTT (SEQ ID NO:100)
CACCAGGAC**G**ACATT (SEQ ID NO:101)
CCACCAGGAC**G**ACAT (SEQ ID NO:102)
TCCACCAGGAC**G**ACA (SEQ ID NO:103)

16-mers:

GAC**G**ACATTTATTAGG (SEQ ID NO:104)
GGAC**G**ACATTTATTAG (SEQ ID NO:105)
AGGAC**G**ACATTTATTA (SEQ ID NO:106)
CAGGAC**G**ACATTTATT (SEQ ID NO:107)
CCAGGAC**G**ACATTTAT (SEQ ID NO:108)
ACCAGGAC**G**ACATTTA (SEQ ID NO:109)
CACCAGGAC**G**ACATTT (SEQ ID NO:110)
CCACCAGGAC**G**ACATT (SEQ ID NO:111)
TCCACCAGGAC**G**ACAT (SEQ ID NO:112 )
GTCCACCAGGAC**G**ACA (SEQ ID NO:113)

17-mers:

GAC**G**ACATTTATTAGGG (SEQ ID NO:114)
GGAC**G**ACATTTATTAGG (SEQ ID NO:115)
AGGAC**G**ACATTTATTAG (SEQ ID NO:116)

CAGGAC**G**ACATTTATTA (SEQ ID NO:117)

CCAGGAC**G**ACATTTATT (SEQ ID NO:118)

ACCAGGAC**G**ACATTTAT (SEQ ID NO:8)

CACCAGGAC**G**ACATTTA (SEQ ID NO:119)

CCACCAGGAC**G**ACATTT (SEQ ID NO:120)

TCCACCAGGAC**G**ACATT (SEQ ID NO:121)

GTCCACCAGGAC**G**ACAT (SEQ ID NO:122)

TGTCCACCAGGAC**G**ACA (SEQ ID NO:123)

18-mers:

GAC**G**ACATTTATTAGGGG (SEQ ID NO:124)

GGAC**G**ACATTTATTAGGG (SEQ ID NO:125)

AGGAC**G**ACATTTATTAGG (SEQ ID NO:126)

CAGGAC**G**ACATTTATTAG (SEQ ID NO:127)

CCAGGAC**G**ACATTTATTA (SEQ ID NO:128)

ACCAGGAC**G**ACATTTATT (SEQ ID NO:129)

CACCAGGAC**G**ACATTTAT (SEQ ID NO:130)

CCACCAGGAC**G**ACATTTA (SEQ ID NO:131)

TCCACCAGGAC**G**ACATTT (SEQ ID NO:132)

GTCCACCAGGAC**G**ACATT (SEQ ID NO:133)

TGTCCACCAGGAC**G**ACAT (SEQ ID NO:134)

CTGTCCACCAGGAC**G**ACA (SEQ ID NO:135)

19-mers:

GAC**G**ACATTTATTAGGGGA (SEQ ID NO:136)

GGAC**G**ACATTTATTAGGGG (SEQ ID NO:137)

AGGAC**G**ACATTTATTAGGG (SEQ ID NO:138)

CAGGAC**G**ACATTTATTAGG (SEQ ID NO:139)

CCAGGAC**G**ACATTTATTAG (SEQ ID NO:140)

ACCAGGAC**G**ACATTTATTA (SEQ ID NO:141)

CACCAGGAC**G**ACATTTATT (SEQ ID NO:142)

CCACCAGGAC**G**ACATTTAT (SEQ ID NO:143)

TCCACCAGGAC**G**ACATTTA (SEQ ID NO:144)

GTCCACCAGGAC**G**ACATTT (SEQ ID NO:145)

TGTCCACCAGGAC**G**ACATT (SEQ ID NO:146)

CTGTCCACCAGGAC**G**ACAT (SEQ ID NO:147)

TCTGTCCACCAGGAC**G**ACA (SEQ ID NO:148)

20-mers:

GAC**G**ACATTTATTAGGGGAC (SEQ ID NO:149)

GGAC**G**ACATTTATTAGGGGA (SEQ ID NO:150)

AGGAC**G**ACATTTATTAGGGG (SEQ ID NO:151)

CAGGAC**G**ACATTTATTAGGG (SEQ ID NO:152)

CCAGGAC**G**ACATTTATTAGG (SEQ ID NO:153)

ACCAGGAC**G**ACATTTATTAG (SEQ ID NO:154)

CACCAGGAC**G**ACATTTATTA (SEQ ID NO:155)

CCACCAGGAC**G**ACATTTATT (SEQ ID NO:156)

TCCACCAGGAC**G**ACATTTAT (SEQ ID NO:157)

GTCCACCAGGAC**G**ACATTTA (SEQ ID NO:158)

TGTCCACCAGGAC**G**ACATTT (SEQ ID NO:159

CTGTCCACCAGGAC**G**ACATT (SEQ ID NO:160)

TCTGTCCACCAGGAC**G**ACAT (SEQ ID NO:161)

ATCTGTCCACCAGGAC**G**ACA (SEQ ID NO:162)

21-mers:

GAC**G**ACATTTATTAGGGGACG (SEQ ID NO:163)

GGAC**G**ACATTTATTAGGGGAC (SEQ ID NO:164)

AGGAC**G**ACATTTATTAGGGGA (SEQ ID NO:165)

CAGGAC**G**ACATTTATTAGGGG (SEQ ID NO:166)

CCAGGAC**G**ACATTTATTAGGG (SEQ ID NO:167)

ACCAGGAC**G**ACATTTATTAGG (SEQ ID NO:168)

CACCAGGAC**G**ACATTTATTAG (SEQ ID NO:169)

CCACCAGGAC**G**ACATTTATTA (SEQ ID NO:170)
TCCACCAGGAC**G**ACATTTATT (SEQ ID NO:171)
GTCCACCAGGAC**G**ACATTTAT (SEQ ID NO:172)
TGTCCACCAGGAC**G**ACATTTA (SEQ ID NO:173)
CTGTCCACCAGGAC**G**ACATTT (SEQ ID NO:174)
TCTGTCCACCAGGAC**G**ACATT (SEQ ID NO:175)
ATCTGTCCACCAGGAC**G**ACAT (SEQ ID NO:176)
TATCTGTCCACCAGGAC**G**ACA (SEQ ID NO:177)

The region of sample DNA on which ASO hybridization is performed to detect the mutation of this invention is preferably amplified by PCR using a forward primer, SEQ ID NO:5, and a reverse primer, SEQ ID NO:6. This amplification yields a 462 nucleotide product within which the mutated site is located. Alternatively, the mutation can be detected using cloned DNA that includes the region of interest, specifically, the region of DNA including position 16,263 of SEQ ID NO:1, and sequencing the DNA. In this case, amplification by PCR or a comparable method is not necessary but can optionally be performed.

Optionally, one or more than one of the amplified regions described above, (including the 462 nucleotide region generated using primers of SEQ ID NO:5 and 6, and the 194 nucleotide region generated using primers of SEQ ID NO:3 and 4), or shorter portions of either of these regions, can be analyzed by sequencing in order to detect the mutation. Sequencing can be performed as is routine in the art. The only limitation on choice of the region to be sequenced, in order to identify the presence of the mutation, is that the region selected for sequencing must include the nucleotide that is the subject of the mutation, which is position 16,263 of SEQ ID NO:1 (based on the publicly available sequence). Deletions or insertions upstream of this site could affect whether the mutation appears at position 16,263 but such deletions or insertions would not affect detection of the point mutation using the methods and probes described herein. The size of the region selected for sequencing is not limited except by technical parameters as is known in the art, and longer regions comprising part or all of the 462 or the 194 base regions disclosed herein can be sequenced.

Variations of the methods disclosed above are also suitable for detecting the mutation. For example, in a variation of ASO, the ASO's are given homopolymer tails with terminal

deoxyribonucleotidyl transferase, spotted onto nylon membrane, and covalently bound by UV irradiation. The target DNA is amplified with biotinylated primers and hybridized to the membrane containing the immobilized oligos, followed by detection. An example of this reverse dot blot technique is the INNO-LIPA kit from Innogenetics (Belgium).

The mutation identified herein is related to AMD pathology. With the identification and sequencing of the mutated gene and the gene product, probes and antibodies raised to the gene product can be used in a variety of hybridization and immunological assays to screen for and detect the presence of either a normal or mutated gene or gene product.

Patient therapy through removal or blocking of the mutant gene product, as well as supplementation with the normal gene product by amplification, by genetic and recombinant techniques or by immunotherapy can be achieved. Correction or modification of the defective gene product by protein treatment immunotherapy (using antibodies to the defective protein) or knock-out of the mutated gene is now also possible. Familial AMD could also be controlled by gene therapy in which the gene defect is corrected *in situ* or by the use of recombinant or other vehicles to deliver a DNA sequence capable of expressing the normal gene product, or a deliberately mutated version of the gene product whose effect counterbalances the deleterious consequences of the disease mutation to the affected cells of the patient.

Expression of the mutated gene in heterologous cell systems can be used to demonstrate structure function relationships. Ligating the DNA sequence into a plasmid expression vector to transfect cells is a useful method to test the influence of the mutation on various cellular biochemical parameters. Plasmid expression vectors containing either the entire normal or mutant human or mouse sequence or portions thereof, can be used in *in vitro* mutagenesis experiments which will identify portions of the protein crucial for regulatory function.

The DNA sequence can be manipulated in studies to understand the expression of the gene and its product, and to achieve production of large quantities of the protein for functional analysis, for antibody production, and for patient therapy. Changes in the sequence may or may not alter the expression pattern in terms of relative quantities, tissue-specificity and functional properties.

The protein can be expressed in insect cells using baculoviral vectors, or in mammalian cells using vaccinia virus or specialized eukaryotic expression vectors. For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus (SV 40) promoter in the pSV2 vector or other similar vectors and introduced into cultured eukaryotic cells such as COS cells to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin and mycophenolic acid.

The DNA sequence can be altered using procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences and site-directed sequence alteration with the use of specific oligonucleotides together with PCR.

The cDNA sequence or portions thereof is introduced into eukaryotic expression vectors by conventional techniques. These vectors permit the transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. The endogenous FIBL-6 gene promoter can also be used. Different promoters within vectors have different activities, which alters the level of expression of the cDNA. In addition, certain promoters can also modulate function such as the glucocorticoid-responsive promoter from the mouse mammary tumor virus.

Cell lines can also be produced which have integrated the vector into the genomic DNA. In this manner, the gene product is produced on a continuous basis. Vectors are introduced into recipient cells by various methods including calcium phosphate, strontium phosphate, electroporation, lipofection, DEAE dextran, microinjection, or by protoplast fusion. Alternatively, the cDNA can be introduced by infection using viral vectors. Using the techniques mentioned, the expression vectors containing the FIBL-6 gene or portions thereof can be introduced into a variety of mammalian cells from other species or into non-mammalian cells.

The recombinant expression vector, according to this invention, comprises the selected DNA of the DNA sequences of this invention for expression in a suitable host. The DNA is operatively joined in the vector to an expression control sequence in the recombinant DNA molecule so that normal or mutant protein can be expressed. The expression control sequence

may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be selected from the group consisting of the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of the fd coat protein, early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus, simian virus, 3-phosphoglycerate kinase promoter, yeast acid phosphatase promoters, yeast alpha-mating factors and combinations thereof.

The host cells to be transfected with the vectors of this invention may be from a host selected from the group consisting of yeasts, fungi, insects, mice or other animals or plant hosts or may be human tissue cells. For the mutant DNA sequence, similar systems are employed to express and produce the mutant protein.

Antibodies to epitopes within the protein can be raised to provide information on the characteristics of the proteins. Generation of antibodies enables the visualization of the protein in cells and tissues using Western blotting. In this technique, proteins are separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. These membranes are then incubated in the presence of a primary antibody, washed and incubated with a secondary antibody to detect the protein-primary antibody complex. Following repeated washing, the entire complex is visualized using colorimetric or chemiluminescent methods.

Antibodies to the protein also allow for the use of immunocytochemistry and immunofluorescence techniques in which the proteins can be visualized directly in cells and tissues. This is most helpful in order to establish the subcellular location of the protein and the tissue specificity of the protein.

In order to prepare polyclonal antibodies, fusion proteins containing defined portions or all of the protein may be synthesized in bacteria by expression of corresponding DNA sequences in a suitable cloning vehicle, as described herein. The protein is then purified, coupled to a carrier protein, mixed with Freund's adjuvant (to help stimulate the antigenic response) and injected into rabbits or other suitable animals. Alternatively, protein can be isolated from cultured cells expressing the protein. Following booster injections at bi-weekly intervals, the rabbits or other suitable animals are bled and the sera isolated. Sera are used directly or purified

prior to use, by various methods including affinity chromatography, Protein A-Sepharose, Antigen Sepharose, Anti-mouse-Ig-Sepharose. Sera or purified antibodies are used to probe protein extracts run on a polyacrylamide gel to identify the FIBL-6 protein. Alternatively, antibodies may be obtained by making synthetic peptides corresponding to antigenic portions of the protein and injecting these into rabbits or other suitable animals.

To produce monoclonal antibodies, cells actively expressing the wild type or mutant FIBL-6 protein are cultured or isolated from tissues and the cell membranes isolated. The membranes, extracts, or recombinant protein extracts containing the protein are injected in Freund's adjuvant into mice. After receiving nine injections over a three week period, the mice are sacrificed and their spleens are removed and resuspended in phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which produce antibody of the appropriate specificity. These cells are then fused with a permanently growing myeloma partner cell, and the products of the fusion are plated into a number of tissue culture wells in the presence of a selective agent such as HAT. The wells are screened by ELISA to identify those containing cells making useful antibody and these cells are freshly plated. After a period of growth, these cells are again screened to identify antibody-producing cells. Several cloning procedures are carried out until over 90% of the wells contain single clones which are positive for antibody production. By this procedure, a stable line of monoclonal antibody-producing clones is established. Monoclonal antibody produced by such clones is purified by methods such as affinity chromatography using Protein A Sepharose or ion-exchange chromatography or by variations and combinations of these techniques.

Antibodies may also be used coupled to other compounds or materials for diagnostic and/or therapeutic uses. For example, they may be coupled to radionuclides for imaging and therapy, or to liposomes for the targeting of compounds contained in the liposomes to a specific tissue location.

Transgenic Mouse Model

The creation of a mouse model for AMD is important to the understanding of the disease and for the testing of possible therapies. There are several ways in which to create an animal model for AMD. One strategy is the generation, in the mouse gene, of a specific mutation such

as the herein identified human gene mutation. Secondly, a wild type human gene could be inserted and/or the murine gene could be humanized by homologous recombination. Thirdly, it is possible to insert a mutant (single or multiple) human gene as a genomic or minigene cDNA construct using wild type, mutant or artificial promoter elements. Fourthly, knock-out of the endogenous murine genes may be accomplished by the insertion of artificially modified fragments of the endogenous gene by homologous recombination. The modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lox p sites) recognized by enzymes such as Cre recombinase.

To inactivate the corresponding mouse gene, chemical or x-ray mutagenesis of mouse gametes, followed by fertilization, can be applied. Heterozygous offspring may be identified by Southern blotting to demonstrate loss of one allele by dosage, or failure to inherit one parental allele using RFLP markers.

To create a transgenic mouse, a mutant version of the human or mouse gene can be inserted into a mouse germ line using standard techniques of oocyte microinjection, or transfection or microinjection into stem cells. Alternatively, if it is desired to inactivate or replace the endogenous FIBL-6 gene, homologous recombination using embryonic stem cells may be applied.

For oocyte injection, one or more copies of the mutant or wild type gene can be inserted into the pronucleus of a just-fertilized mouse oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn mice are screened for integrants using analysis of tail DNA for the presence of human gene sequences. The transgene may be either a complete genomic sequence injected as a YAC, BAC, PAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

Retroviral infection of early embryos can also be done to insert the mutant or wild type human gene. In this method, the mutant or wild type gene is inserted into a retroviral vector which is used to infect mouse embryos directly during the early stages of development to generate chimeras, some of which will lead to germline transmission. Similar experiments can

be conducted in the case of mutant proteins, using mutant murine or other animal gene sequences.

Homologous recombination using stem cells allows for the screening of gene transfer cells to identify the rare homologous recombination events. Once identified, these can be used to generate chimeras by injection of mouse blastocysts, and a proportion the resulting mice will show germline transmission from the recombinant line. This methodology is especially useful if inactivation of the mouse gene is desired. For example, inactivation of the mouse gene can be done by designing a DNA fragment which contains sequences from a mouse exon flanking a selectable marker. Homologous recombination leads to the insertion of the marker sequences in the middle of an exon, inactivating the FIBL-6 gene. DNA analysis of individual clones can be used to recognize the homologous recombination events.

It is also possible to create mutations in the mouse germline by injecting oligonucleotides containing the mutation of interest and screening the resulting cells by PCR.

Isolation of FIBL-6 Binding Proteins

Isolation of interacting partners of the FIBL-6 allows identification of the biochemical partners for the FIBL-6 and thus the identification of the biochemical pathway disturbed by mutations in FIBL-6. Such partners could be for example, enzymes, co-receptors, ligands or stabilizers. By analyzing these interactions, it is possible to design compounds which counteract the effect of the mutation interaction, thus providing treatment for abnormal interactions. These treatments might alter the interaction of the FIBL-6 with these partners, they may alter the function of the interacting protein, they may alter the amount or tissue distribution or expression of the interaction partners, or they may alter similar properties of the FIBL-6.

Soluble recombinant fusion proteins can be made in suitable vectors (yeast-2-hybrid, baculovirus, and phage-display systems for instance) and used to identify other proteins which interact with FIBL-6 in the pathogenesis of AMD. Therapies can be designed to modulate these interactions and thus modulate AMD and the other conditions associated with acquired or inherited abnormalities of the FIBL-6 gene or gene products. The potential efficacy of these therapies can be tested by analyzing the affinity and function of these interactions after exposure

to the therapeutic agent by standard pharmacokinetic measurements of affinity (Kd and Vmax etc.) using synthetic peptides or recombinant proteins corresponding to functional domains of the FIBL-6 gene. Another method for assaying the effect of any interactions involving functional domains is to monitor changes in the intracellular trafficking and post-translational modification of the relevant genes by *in situ* hybridization, immunohistochemistry, Western blotting and metabolic pulse-chase labelling studies in the presence of, and in the absence of, the therapeutic agents. A further method is to monitor the effects of "downstream" events including (i) changes in the intracellular metabolism, trafficking and targeting of APP and its products; (ii) changes in second messenger events.

Two-Hybrid Yeast System

The Two-Hybrid system, which is known in the art, takes advantage of transcriptional factors that are composed of two physically separable, functional domains. The most commonly used is the yeast GAL4 transcriptional activator consisting of a DNA binding domain and a transcriptional activation domain. Two different cloning vectors are used to generate separate fusions of the GAL4 domains to genes encoding potential binding proteins. The fusion proteins are co-expressed, targeted to the nucleus and, if interactions occur, activation of a reporter gene (e.g. lacZ) produces a detectable phenotype.

Identification of Small Molecules with FIBL-6 Binding Capacity

Small molecule-based therapies are particularly preferred because such molecules are more readily absorbed after oral administration, and have fewer potential antigenic determinants than larger, protein-based pharmaceuticals. In light of the present disclosure, one of ordinary skill in the art is enabled to develop drug screening methodologies which will be useful in the identification of candidate small molecule pharmaceuticals for the treatment of AMD. In particular, one is enabled to screen large libraries of small molecules in order to identify those which bind to the normal and/or mutant protein and which, therefore, are candidates for modifying the *in vivo* activity of the normal or mutant FIBL-6 proteins. Furthermore, one is enabled to identify small molecules which selectively or preferentially bind to a mutant form of a protein and which, therefore, may have particular utility in treating heterozygous carriers of this disease.

Methods for screening small molecule libraries for candidate protein-binding molecules are well known in the art and, in light of the present disclosure, may now be employed to identify compounds which bind to the normal or mutant forms of FIBL-6. Briefly, in one embodiment, either a normal or mutant FIBL-6 protein may be immobilized on a substrate such as a column or filter, and a solution including the test compound(s) is contacted with the FIBL-6 protein under conditions which are permissive for binding. The substrate is then washed with a solution which substantially reflects physiological conditions to remove unbound or weakly bound small molecules. A second wash may then elute those compounds which strongly bound to the immobilized normal or mutant FIBL-6. Alternatively, the small molecule test compounds may be immobilized and a solution of normal or mutant FIBL-6 may be contacted with the column, filter or other substrate. The ability of the FIBL-6 to bind to the small molecules may be determined as above or a labelled form of FIBL-6 (e.g., radio-labelled or chemiluminescent) may be used to more rapidly assess binding to the substrate immobilized compound(s). In addition, as FIBL-6 is believed to be a membrane associated protein, it may be preferred that the FIBL-6 protein be incorporated into lipid bilayers (e.g., liposomes) to promote its proper folding. Such FIBL-6-liposomes may be immobilized on substrates (either directly or by means of another element in the liposome membrane), passed over substrates with immobilized small molecules, or used in any of a variety of other well known binding assays for membrane proteins.

In another series of embodiments, either normal or mutant, free or membrane-bound FIBL-6 may be mixed in a solution with the candidate compound(s) under conditions which are permissive for binding, and the FIBL-6 may be immunoprecipitated. Small molecules which co-immunoprecipitate with a FIBL-6 may then be identified. As will be obvious to one of ordinary skill in the art, there are numerous other methods of screening individual small molecules or large libraries of small molecules (e.g., phage display libraries) to identify compounds which bind to normal or mutant FIBL-6. All of these methods comprise the step of mixing normal or mutant FIBL-6 with test compounds, allowing for binding (if any), and assaying for bound complexes. All such methods are now enabled by the present disclosure of the mutant FIBL-6 protein.

Because the normal physiological roles of FIBL-6 is still unknown, compounds which bind to normal or mutant or both forms of FIBL-6 may have utility in treatments. Compounds which bind only to a normal FIBL-6 may, for example, act as enhancers of its normal activity and thereby at least partially compensate for the lost or abnormal activity of mutant forms of the FIBL-6 in AMD patients. Compounds which bind to both normal and mutant forms of a FIBL-6 may have utility if they differentially affect the activities of the two forms so as to alleviate the overall departure from normal function. Alternatively, blocking the activity of both normal and mutant forms of FIBL-6 in heterozygotes may have less severe physiological and clinical consequences than the normal progress of the disease and, therefore, compounds which bind to and inhibit the activity of both normal and mutant forms of a FIBL-6 may have utility. Preferably, however, compounds are identified which have a higher affinity of binding to mutant FIBL-6 than to normal FIBL-6 (e.g., 5-10 fold higher $K_a$) and which selectively or preferentially inhibit the activity of the mutant form. Such compounds may be identified by using any of the techniques described above and by then comparing the binding affinities of the candidate compound(s) for the normal and mutant forms of FIBL-6.

Once identified by the methods described above, the candidate compounds may then be produced in quantities sufficient for pharmaceutical administration or testing (e.g., μg or mg or greater quantities), and formulated in a pharmaceutically acceptable carrier (see, e.g., Remington's Pharmaceutical Sciences, Gennaro, A., ed., Mack Pub., 1990, the disclosure of which is incorporated herein by reference). These candidate compounds may then be administered to AMD patients or animal models of AMD. The animal models described and enabled herein are of particular utility in further testing all candidate molecules which bind to normal or mutant FIBL-6 for their therapeutic efficacy.

Once identified by the methods described above, the candidate compounds may also serve as "lead compounds" in the design and development of new pharmaceuticals. For example, as is well known in the art, sequential modification of small molecules (e.g., amino acid residue replacement with peptides, functional group replacement with peptide or non-peptide compounds) is a standard approach in the pharmaceutical industry for the development of new pharmaceuticals. Such development generally proceeds from a "lead compound" which is shown to have at least some of the activity (e.g., FIBL-6 binding ability) of the desired

pharmaceutical. In particular, when one or more compounds having at least some activity of interest (e.g., FIBL-6 binding) are identified, structural comparison of the molecules can greatly inform the skilled practitioner by suggesting portions of the lead compounds which should be conserved and portions which may be varied in the design of new candidate compounds. Thus, the present invention also provides a means of identifying lead compounds which may be sequentially modified to produce new candidate compounds for use in the treatment of AMD. These new compounds then may be tested both for FIBL-6-binding (e.g., in the binding assays described above) and for therapeutic efficacy (e.g., in the animal models described herein). This procedure may be iterated until compounds having the desired therapeutic activity and/or efficacy are identified.

Screening and Diagnosis for AMD

General Diagnostic Methods

The FIBL-6-related genes and gene products, as well as other products derived therefrom (e.g., probes, antibodies), will be useful in the diagnosis of AMD. Diagnosis of inherited cases of these diseases can be accomplished by methods based upon the nucleic acids (including genomic and mRNA/cDNA sequences), proteins, and/or antibodies disclosed and enabled herein. Preferably, the methods and products are based upon the human FIBL-6 nucleic acids, proteins or antibodies disclosed herein. As will be obvious to one of ordinary skill in the art, however, the significant evolutionary conservation of the mutated region of the FIBL-6 nucleotide and amino acid sequences allows the skilled artisan to make use of non-human FIBL-6-homologue nucleic acids, proteins and antibodies even for applications directed toward human or other mammalian subjects.

As will be appreciated by one of ordinary skill in the art, the choice of diagnostic methods of the present invention will be influenced by the nature of the available biological samples to be tested and the nature of the information required. Assays based upon a subject's genomic DNA may be the preferred methods for FIBL-6 diagnostics as no information will he lost due to alternative splicing and because essentially any nucleate cells may provide a usable sample. Diagnostics based upon other FIBL-6-related proteins are subject to similar

considerations: availability of tissues, levels of expression in various tissues, and alternative translation products resulting from alternative mRNA splicing.

When a diagnostic assay is to be based upon FIBL-6-related proteins, a variety of approaches are possible. For example, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the molecular mass of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products. In some preferred embodiments, protein-based diagnostics will employ differences in the ability of antibodies to bind to normal and mutant FIBL-6-related proteins. Such diagnostic tests may employ antibodies which bind to the normal proteins but not to mutant proteins, or vice versa. Because a specific point mutation has been identified in the FIBL-6 gene/protein, and because this mutation appears to be conserved, it is currently preferred that antibodies capable of selectively binding to mutant proteins be employed. In particular, an assay using a plurality of monoclonal antibodies, each capable of binding to a mutant epitope, may be employed. The levels of anti-mutant antibody binding in a sample obtained from a test subject (visualized by, for example, radiolabelling, ELISA or chemiluminescence) may be compared to the levels of binding to a control sample. Such antibody diagnostics may be used for *in situ* immunohistochemistry using biopsy samples of retinal tissues obtained antemortem or postmortem.

When the diagnostic assay is to be based upon nucleic acids from a tissue sample, either mRNA or genomic DNA may be used. When mRNA is used from a sample, many of the same considerations apply with respect to source tissues and the possibility of alternative splicing. That is, there may be little or no expression of transcripts unless appropriate tissue sources are chosen or available, and alternative splicing may result in the loss of some information. With either mRNA or DNA, standard methods well known in the art may be used to detect the presence of a particular sequence either *in situ* or *in vitro* (see, e.g. Sambrook et al., eds. (1989)

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.).

For *in situ* detection of a mutant FIBL-6, or other FIBL-6-related nucleic acid sequence, a sample of tissue, which can include skin, may be prepared by standard techniques and then contacted with a probe, preferably one which is labelled to facilitate detection, and an assay for nucleic acid hybridization is conducted under stringent conditions which permit hybridization only between the probe and highly or perfectly complementary sequences. Because the FIBL-6 mutation detected to date consists of a single nucleotide substitution, high stringency hybridization conditions will be required to distinguish normal sequences from most mutant sequences. As an example only, the following procedure may be employed on a subject: A rat animal model is anesthetized and transcardially perfused with cold PBS, followed by perfusion with a formaldehyde solution. The tissue of interest is then removed, frozen in liquid nitrogen, and cut into thin micron sections. The sections are placed on slides and incubated in proteinase K. Following rinsing in DEP, water and ethanol, the slides are placed in prehybridization buffer. A radioactive probe corresponding to the selected oligonucleotide is incubated with the sectioned tissue. After incubation and air drying, the labeled areas are visualized by autoradiography. Dark spots on the tissue sample indicate hybridization of the probe with tissue mRNA, demonstrating expression of the nucleic acid sequence.

A significant advantage of the use of either DNA or mRNA is the ability to amplify the amount of genetic material using the polymerase chain reaction (PCR), either alone (with genomic DNA) or in combination with reverse transcription (with mRNA to produce cDNA). It is contemplated that such PCR-based genetic methods may be preferred commercial embodiments for diagnostic screenings.

Screening for AMD Linked to Chromosome 1q

Screening for AMD as linked to chromosome 1q may now be readily carried out because of the knowledge of a mutation in the gene provided by this invention.

Individuals with a high risk for AMD (present in family pedigree), or individuals not previously known to be at risk, or people in general may be screened routinely using probes to

detect the presence of a mutant FIBL-6 gene by a variety of techniques. Genomic DNA used for the diagnosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be PCR amplified prior to analysis. RNA or cDNA may also be used. To detect a specific nucleic acid sequence, hybridization using specific oligonucleotides, direct nucleotide sequencing, restriction enzyme digest, RNase protection, chemical cleavage, or ligase-mediated detection may be used. Oligonucleotides specific to mutant sequences can be chemically synthesized and labelled radioactively with isotopes, or non-radioactively using biotin tags, and hybridized to individual samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these mutant sequences is then visualized using methods such as autoradiography, fluorometry, or colorimetric reaction. Examples of suitable PCR primers which are useful for example in amplifying portions of the subject sequence containing the aforementioned mutations are set out in the Examples. Direct DNA sequencing reveals sequence differences between normal and mutant FIBL-6 DNA. Cloned genomic or cDNA segments may be used as probes to detect specific DNA segments. PCR can be used to enhance the sensitivity of this method. PCR, which is well-known in the art, is an enzymatic amplification directed by sequence-specific primers, and involves repeated cycles of heat denaturation of the DNA, annealing of the complementary primers and extension of the annealed primer with a DNA polymerase. This results in an exponential increase of the target DNA.

Other nucleotide sequence amplification techniques may be used, such as ligation-mediated PCR, anchored PCR and enzymatic amplification as will be understood by those skilled in the art.

Sequence alterations may also generate restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by gel-blot hybridization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or decrease of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme and the fragments of different sizes are visualized, for example under UV light in the presence of ethidium bromide, after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis of single stranded DNA, or as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis. Alternatively, a single base substitution mutation may be detected based on differential PCR product length in PCR. The PCR products of the normal and mutant gene may be differentially detected in acrylamide gels. Nuclease protection assays (S1 or ligase-mediated) also reveal sequence changes at specific locations.

Alternatively, to confirm or detect a polymorphism, ligated PCR, ASO, REF-SSCP chemical cleavage, endonuclease cleavage at mismatch sites or SSCP may be used. Both REF-SSCP and SSCP are mobility shift assays which are based upon the change in conformation due to mutations.

DNA fragments may also be visualized by methods in which the individual DNA samples are not immobilized on membranes. The probe and target sequences may be in solution or the probe sequence may be immobilized. Autoradiography, radioactive decay, spectrophotometry and fluorometry may also be used to identify specific individual genotypes. Mutations can also be detected by direct nucleotide sequencing.

According to an embodiment of the invention, the portion of the cDNA or genomic DNA segment that is informative for a mutation can be amplified using PCR. For example, the DNA segment immediately surrounding the Gln5345Arg mutation acquired from any cell sample from an individual, such as RPE, epithelial cell, can be screened using the oligonucleotide primers SEQ ID NO:3 and SEQ ID NO:4. This region would then be amplified by PCR, the products separated by electrophoresis, and transferred to membrane. Normal and mutant PCR products may then be detected using, for example, hybridization of labeled oligonucleotide probes and autoradiography, RFLP analysis, or direct sequencing. In inherited cases, as the primary event, and in non-inherited cases as a secondary event due to the disease state, abnormal processing of FIBL-6 or proteins reacting with FIBL-6 may occur. This can be detected as abnormal phosphorylation, glycosylation, glycation amidation, or proteolytic cleavage products in body tissues or fluids.

Diagnosis of non-inherited cases also can be made by observation of alterations in the FIBL-6 transcription, translation, and post-translational modification and processing as well as alterations in the intracellular and extracellular trafficking of FIBL-6 gene products in the RPE or epithelial cells. Such changes will include alterations in the amount of FIBL-6 messenger RNA and/or protein; alteration in phosphorylation state, abnormal intracellular location/distribution, abnormal extracellular distribution, etc. Such assays will include: Northern Blots (with FIBL-6-specific and non-specific nucleotide probes which also cross-react with other members of the gene family), and Western blots and enzyme-linked immunosorbent assays (ELISA) (with antibodies raised specifically to: a FIBL-6; to various functional domains of a FIBL-6; to other members of the homologous gene family; and to various post-translational modification states including glycosylated and phosphorylated isoforms). These assays can be performed on peripheral tissues (e.g. skin, blood cells, plasma, cultured or other fibroblast tissues, etc.) as well as on biopsies of retinal tissues obtained antemortem or postmortem. Such assays might also include *in situ* hybridization and immunohistochemistry to localized messenger RNA and protein to specific subcellular compartments and/or within structures associated with these diseases.

In accordance with the present invention, diagnostic kits are also provided which will include the reagents necessary for the above-described diagnostic screens. For example, kits may be provided which include antibodies or sets of antibodies which are specific to one or more mutant epitopes. These antibodies may, in particular, be labelled by any of the standard means which facilitate visualization of binding. Alternatively, kits may be provided in which oligonucleotide probes or PCR primers are present for the detection and/or amplification of mutant FIBL-6, and other FIBL-6-related nucleotide sequences. Again, such probes may be labelled for easier detection of specific hybridization. As appropriate to the various diagnostic embodiments described above, the oligonucleotide probes or antibodies in such kits may be immobilized to substrates and appropriate controls may be provided. Examples of such probes include oligonucleotides comprising or consisting of at least one of SEQ ID NO:7, SEQ ID NO:8. SEQ ID NO:12-94, and SEQ ID NO:95-177.

Therapies

An important aspect of the biochemical studies using the genetic information of this invention is the development of therapies to circumvent or overcome the FIBL-6 gene defect, and thus prevent, treat, control serious symptoms or cure the disease. AMD manifests itself as a vision-related disorder which in one of its forms is associated with an inherited mutation in a FIBL-6 gene.

Protein Therapy

Treatment of AMD can be performed by replacing the mutant protein with normal protein, or by modulating the function of the mutant protein. Once the biological pathway of the involved FIBL-6 protein has been completely understood, it may also be possible to modify the pathophysiologic pathway or pathways (e.g. a signal transduction pathway) in which the protein participates, in order to correct the physiological defect.

To replace the mutant protein with normal protein, or with a protein bearing a deliberate counterbalancing mutation, it is necessary to obtain large amounts of pure FIBL-6 protein from cultured cell systems which can express the protein. Delivery of the protein to the affected eye tissue or other tissues can then be accomplished using appropriate packaging or administrating systems.

Gene Therapy

Gene therapy is another potential therapeutic approach in which normal copies of the FIBL-6 gene are introduced into patients to code successfully for normal protein in affected cell types. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Alternatively, in some mutants it has been possible to prevent disease by introducing another copy of the homologous gene bearing a second mutation in that gene or to alter the mutation, or use another gene to block its effect.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression levels of normal protein should be high enough to

achieve the desired effect. The full length FIBL-6 gene can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest. Other viral vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr virus.

Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are of lower efficiency.

Antisense based strategies can be employed to explore FIBL-6 gene function and as a basis for therapeutic drug design. The principle is based on the hypothesis that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary antisense species. The formation of a hybrid RNA duplex may then interfere with the processing, transport, translation, and/or stability of the target FIBL-6 mRNA. Hybridization is required for the antisense effect to occur, but the efficiency of intracellular hybridization can be low and therefore the consequences of such an event may not be very successful. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides, injection of antisense RNA and transfection of antisense RNA expression vectors. Antisense effects can be induced by control (sense) sequences, however, the extent of phenotypic changes are highly variable. Phenotypic effects induced by antisense effects are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

Transplantation of normal genes into the affected area of the patient can be useful therapy for AMD. In this procedure, a normal human FIBL-6 gene is transferred into a cultivatable cell type, either exogenously or endogenously to the patient. These cells are then injected serotologically into the disease-affected tissue or tissues.

Immunotherapy is also possible for AMD. Antibodies are raised to a mutant FIBL-6 protein (or a portion thereof) and are administered to the patient to bind or block the mutant protein and prevent its deleterious effects. Simultaneously, expression of the normal protein

product could be encouraged. Alternatively, antibodies are raised to specific complexes between mutant or wild-type FIBL-6 and their interaction partners.

A further approach is to stimulate endogenous antibody production to the desired antigen. Administration could be in the form of a one time immunogenic preparation or vaccine immunization. An immunogenic composition may be prepared as injectables, as liquid solutions or emulsions. The FIBL-6 or antigen may be mixed with pharmaceutically acceptable excipients compatible with the protein. Such excipients may include water, saline, dextrose, glycerol, ethanol and combinations thereof. The immunogenic composition and vaccine may further contain auxiliary substances such as emulsifying agents or adjuvants to enhance effectiveness. Immunogenic compositions and vaccines may be administered parenterally by injection subcutaneously or intramuscularly.

The immunogenic preparations and vaccines are administered in such amount as will be therapeutically effective, protective and immunogenic. Dosage depends on the route of administration and will vary according to the size of the host.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in the form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

## EXAMPLE 1

### Identification of a Family Carrying an AMD-Associated Gene

#### Clinical Information

Twenty-one individuals from a family with AMD agreed to participate in the original study, which was approved by the Institutional Review Board of Oregon Health Sciences University, Portland. For all but one individual, stereoscopic fundus photographs of the macula

of both eyes were obtained. For the remaining individual, now deceased, the eyes were retrieved for histopathologic analysis. Clinical information, including visual acuity, was obtained for all affected individuals. In most instances, this information was obtained from the individual's local ophthalmologist or optometrist, and fundus photographs were taken at a local retina facility.

Classification of AMD

Stereoscopic fundus photographs of the macula were classified using a modified version of the Wisconsin Age-Related Maculopathy Grading System. Each eye was classified independently by three ophthalmologists without knowledge of the patient's genotype. Disagreements were adjudicated among the three graders. The patient's classification was based on the eye with the more advanced AMD.

The following classification system was used: Group 1, definite AMD is characterized by exudative AMD (choroidal neovascularization, pigment epithelial detachment, or disciform macular scar) or geographic atrophy (area, >175 μm). Group 2, probably AMD, is characterized by the presence of large drusen (>125 μm) within 3000 μm of the fovea, with total cumulative drusen area exceeding 393,744 $\mu m^2$ (approximating the area within a 700-μm diameter circle). Nongeographic pigment atrophy and focal hyperpigmentation may or may not be present, and the features present in group 1 are absent. Group 3, probably no AMD, is characterized by large drusen present (>125 μm), but the cumulative area is less in extent than in group 2. Group 3 is characterized by the absence of exudative AMD, geographic or nongeographic pigment atrophy, and local hyperpigmentation. Group 4, no AMD, is characterized by no large drusen (>125 μm), geographic or nongeographic pigment atrophy, focal hyperpigmentation, or exudative maculopathy. Group 5, uncertain, is characterized by absence of features seen in groups 1 and 2 and presence of any of the following: extensive small (<63 μm) or intermediate (63-125 μm) drusen, nongeographic pigment atrophy, or focal hyperpigmentation, or factors preventing reliable classification, such as media opacities, concomitant retinal disease, or pigment epithelial disturbance, with or without large drusen (>125 μm).

The definition of group 2 was based on findings demonstrating a 19-fold increase in the probability of developing late AMD (group 1) for eyes with large drusen (>125 μm) and a minimum cumulative drusen area of 393,744 μm (equivalent to an approximately 700-μm

diameter circle) located within 3,000 μm of the fovea. Eyes classified as group 5, uncertain, are those in which accompanying conditions preclude accurate classification of AMD or eyes that contain possible risk factors for the later development of late AMD, including extensive small and intermediate drusen (<125 μm) or pigment epithelial abnormalities.

For purposes of linkage analysis, groups 1 and 2 were classified as affected and groups 3 and 4 were classified as unaffected. Those categorized as group 5 were classified as unknowns in the linkage analysis.

Genotyping

Genomic DNA was extracted from the blood of family members using a kit from Epicentre Technologies (Madison, WI) according to their directions and was quantitiated spectrophotometrically. Three pools of DNA were created. The first pool consisted of equimolar amounts of DNA from eight affected family members (patients 1009, 2101, 1011, 1015, 1007, 2705, 1017, and 2005). The second pool was similarly derived from four unaffected family members (patients 2009, 2502, 2011, and 2103). These pools represented all affected and unaffected individuals who had been ascertained as the pooling was initiated. A third pool consisted of equimolar amounts of DNA from eight unrelated individuals. Three affected cousins from the family with AMD (patients 2101, 2705, and 2005) were also individually genotyped. 615 polymorphic microsatellite repeat markers at an average density of six centimorgans (cM) were used to genotype DNA from the three selected individuals and the three DNA pools. 572 of these markers yielded clearly identifiable banding patterns. After the initial pooled genome-wide screen, markers from areas most suggestive of shared chromosomal segments were used to genotype all potentially informative family members. One region showing positive linkage was then mapped at an average marker density of 0.5 cM.

Linkage Analysis

Two-point linkage between the disease locus and each microsatellite marker locus was tested by the parametric lodscore method using a computer program (MLINK). Frequencies of the disease allele and of the normal alleles were assumed to be 0.001 and 0.999, respectively. Based on the pedigree in which there were 3 generations of affected individuals, and male-to-male transmission, an autosomal-dominant mode of inheritance was assumed. Family members

were placed in 1 of 5 age-related liability classes. Age-dependent penetrances for these classes were set to 0.001 (<50 years), 0.01 (50-54 years), 0.09 (55-64 years), 0.42 (65-74 years), and 0.95 (>75 years). These values were determined from a set of 20 similar families with AMD that the inventors identified and are comparable with the prevalence observations reported in three studies based on approximately 15,000 individuals. Allele frequencies reported by the Centre d'Etudes du Polymorphisme Humain (CEPH), Paris, France (http://www.cephb.fr/cephdh/), were used except for markers D15191, D15202, D15461, D15492, and D15412, which were measured in a set of 92 unrelated individuals. Markers for multipoint linkage analysis, whose order was statistically supported, were identified using genotypes from CEPH pedigrees and the computer programs CRI-MAP and MultiMap. Multipoint linkage analysis was conducted using the VITESSE algorithm.

## EXAMPLE 2

### Screening Family for AMD-Associated Mutation

#### Subject ascertainment

Human subjects were informed and consented as mandated by the Institutional Review Board of Oregon Health & Science University. Approximately 20 ml of blood was collected from each subject and DNA was extracted by standard techniques. In total, 100 families with three or more members affected with AMD, 188 sporadic cases of AMD, and 174 phenotypically normal individuals were studied. The diagnosis of AMD was based upon stereoscopic fundus photographs as previously described. (Klein ML, *Arch Ophthalmol* 116:1082-8, 1998.)

#### Mutation screening

Two monoallelic strains (human-mouse cell hybrids), derived from proband III-3 of family A, were generated by GMP Genetics, Inc. (Waltham, MA). One carried the *ARMD1* region corresponding to the disease haplotype and the other, a presumably non-disease sequence. Inclusion of the *ARMD1* gene locus in the hybrids was verified in the laboratory by genotyping with six microsatellite markers. Exons were mapped on genomic sequence through a pairwise comparison (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) of XM_053531, the reference sequence for FIBL-6 mRNA, with contigs AL121996, AL135796, AL133515, AL391824,

AL118512, AL135797, and AL133553. Primers were designed to amplify each exon plus an additional 50-100 basepairs of the adjacent introns using the Primer3 software package (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Genomic DNA (25 ng) was amplified in 20 μl reactions using FastStart Taq DNA polymerase (Roche Diagnostic Corporation Indianapolis, IN) with a balcony PCR protocol, which helped to prevent amplification of homologous mouse DNA. All amplifications included an initial denaturation at 95° C for 5 minutes, a 30-second denaturation at 95° C, an annealing step, and a 1-minute extension at 72° C. "Balcony" refers to the first 10 cycles, in which annealing was performed at 5° C above the $T_m$ of the primers as determined by Primer3 software. This was followed by a 20 cycle touchdown phase in which the annealing temperature dropped in 0.5° C increments from 5° C above the primer $T_m$ to 5° C below. The final phase consisted of 25 cycles in which annealing occurred at 5° C below primer $T_m$. PCR products were electrophoresed on 2% agarose gels, excised, purified with Microcon-PCR Filter units (Millipore Corporation, Bedford, MA), and sequenced at the Veteran's Administration Core Sequencing facility (Portland, OR).

Control subjects (n=174), sporadic AMD cases (n=188), and family members (n=1016) were assayed for the Gln5345Arg variation by DHPLC (Transgenomics, Omaha, NE) or by ASO hybridization or both. For DHPLC, a 194 bp PCR product, located within exon 104 of *FIBL-6,* was amplified using the forward primer, 5'CCGTGCAAGGTTATAGCTACTG3' (SEQ ID NO:3), and the reverse primer, 5'ATGGCATACGAGCAGACATT3' (SEQ ID NO:4). In order to increase the sensitivity of detecting potential homozygous mutations, samples were mixed 1:1 with wildtype product prior to a final 5-minute denaturation and slow reannealing step. For ASO hybridization, a 462 bp product was amplified using the forward primer, 5'TATCATGGCATACGAGCAGAC3' (SEQ ID NO:5), and the reverse primer, 5'TTCACTGCACTCAAACAATCAC3' (SEQ ID NO:6). Blotting and hybridization were performed as previously described, Litt M, Kramer P, LaMorticella DM, Murphey W, Lovrien EW, Weleber RG, *Hum Mol Genet* 7:471-4, 1998, except that blots were probed at 47 °C overnight with a 17 base wildtype oligomer (16,263**A**), 5'ACCAGGAC**A**ACATTTAT3' (SEQ ID NO:7), or mutant oligomer (16,263**G**), 5'ACCAGGAC**G**ACATTTAT3' (SEQ ID NO:8).

RT-PCR of human cell lines

Human RPE cells and skin fibroblasts were cultured as previously detailed. Alexander JP, Bradley JMB, Gabourel JD, Acott TS, *Investigative Ophthalmology & Visual Science* 31:2520-8, 1990; Vranka JA, *Current Eye Research* 16:102-110, 1997; Alexander JP, Samples JR, Van Buskirk EM, Acott TS, *Investigative Ophthalmology & Visual Science* 32:172-80, 1991. Total RNA was extracted from cultured cells and various human ocular tissues using an RNAqueous kit (Ambion). RT-PCR utilized an RNA Amplification kit (Roche) in a Roche LightCycler with detection after each cycle based on SybrGreen binding. Two sets of primers were used to differentiate alternative splicing involving exon 104. A common upstream primer, 5'-CAAGAAGCAGCTATCGTTGTG-3' (SEQ ID NO:9), was located approximately 100 bp proximal to the 3' end of exon 103. One downstream primer, 5'-ACTGTCTGTAATGCTGTTGAG GT-3' (SEQ ID NO:10), was located within exon 104 and produced a 297 bp PCR product when exon 104 was present in the transcript. The other primer, 5'-GCATGTCTTTCCATTGTGTGT-3' (SEQ ID NO:11), was located approximately 100 bp downstream of the 5' end of exon 105 and yielded products of 536 bp or 185 bp depending on the presence or absence of exon 104 in the transcript. Several resultant PCR products were gel-purified and sequenced for further verification.

Sequencing of additional species

*Oryctolagus cuniculus* cDNA was obtained from the Casey Eye Institute, Oregon Health & Science University (OHSU). *Canis familiaris*, *Felis catus*, *Ovis sp.*, and *Rattus norvegicus* blood was obtained from the Department of Comparative Medicine, OHSU. A chicken cell line was obtained from the Pathology Department, OHSU. Porcine tissues were obtained from Carlton Packing (Carlton, OR). Three sets of primers were designed to the most conserved regions between mouse and human copies of exon 104 of *FIBL-6* that included the Gln5345 codon. Amplifications used less stringent conditions than normal to compensate for potential non-complementarity between primers and template. An initial, "touchup" phase of PCR included 8-20 cycles, in which the annealing temperature was raised incrementally from 10-15°C below the primer $T_m$ to 5-10 °C below. This was followed by 25 cycles of PCR in which annealing occurred at 5-10 °C below the primer $T_m$. The magnesium concentration was also increased to 2-4 mM. If the initial PCR product was weak or not unique, the amplification was

repeated with a 1:1000 dilution of product as template under normal stringent conditions (annealing at 5 °C below $T_m$, 1.5 mM magnesium concentration).

According to this Example, a potential disease-causing variation (A16,263G) in exon 104 of *FIBL-6* was identified by comparing the DNA sequence of two monoallelic clones (Yan H, *Nature* 403:723-4, 2000) obtained from the proband (III-3) of family A (Figure 3). This sequence variation is expected to change the glutamine at amino acid position 5345 of FIBL-6 to arginine (Gln5345Arg). Since this was a non-conservative amino acid substitution, DNA from all the members of family A was sequenced to determine if the variation segregated with the disease haplotype.

The Gln5345Arg variation segregated exclusively with the disease locus in family A (Figure 4). It is not associated with any of the seventeen other haplotypes across the *ARMD1* region found in this pedigree. DNA sequencing revealed that all sixteen family members who carried the disease haplotype also carried the Gln5345Arg variation, including the ten affected members described previously. Klein ML, *Arch Ophthalmol* 116:1082-8, 1998. Furthermore, none of the eleven family members who lack the disease haplotype carried the variation.

The glutamine at position 5345 of FIBL-6 appears to be conserved among eutherian species. Since the only other *FIBL-6* mammalian DNA sequence deposited at Genbank belonged to mouse, a region was sequenced corresponding to a portion of human exon 104 in pig, rabbit, dog, rat, cat, sheep, and chicken. In all mammals assayed and in chicken, the amino acid at a position equivalent to 5345 in human FIBL-6 was conserved as glutamine (Figure 2). There was approximately 85% conservation at the nucleotide level and approximately 90% conservation at the amino acid level among all mammalian species in the portion of the cbEGF domain in exon 104 sequenced. In mouse and rabbit, this glutamine is encoded by a CAG codon instead of the CAA codon found in other species.

In an initial DHPLC screen of a single affected member from 100 AMD families, we identified the Gln5345Arg variation in two additional pedigrees, families B and C. Sequencing of DNA from all the family members showed that the variation does not segregate with AMD in either family (Figure 5). However, in both families, the Gln5345Arg variation segregated with a haplotype that was inherited apparently from the spouse (II-4 and II-1 in families B and C,

respectively) of a family member. An ASO hybridization screen of all 1016 affected and unaffected family members identified only one additional person with the Gln5345Arg variation. This affected individual, II-4 in family D, also has a unique haplotype across this region that is not shared by any other members of his family (Figure 5).

Individuals from all four families who carry the Gln5345Arg variation share a unique haplotype that overlaps the *FIBL-6* gene. They all have in common an allele of six contiguous markers in the region of *FIBL-6* (Figure 5). Furthermore, three families (A, B, and D) share an allele of an additional four contiguous markers (D1S2138, D1S3460, D1S202, and D1S1642) distal to these for a total of ten. Families C and D also share three contiguous markers (D1S2127, D1S2701, and D1S158) proximal to these six.

Additionally, 2 of 348 chromosomes from control subjects and 1 of 376 chromosomes from sporadic AMD cases were found to harbor the Gln5345Arg variation by DHPLC and ASO hybridization. While the sporadic case was 74 years of age at the time of ascertainment, the control subjects were only 57 and 64 years of age. The 64-year old control subject has a scar in the macula of her left eye. In order to assay for additional variations that may mitigate the effect of the Gln5345Arg variation, all 107 exons of *FIBL-6* from both control subjects were sequenced. The 57-year-old control subject was found to harbor five of the twelve variations detected in the exons of *FIBL-6*. One of these was further investigate, as described below. Although haplotypes are yet to be assigned, the genotypes of these three individuals suggest that the Gln5345Arg variation is associated with the same unique haplotype that is found in all four families (Figure 5).

Few variations were identified among the 16,905 nucleotides and 5,635 amino acid residues that encode FIBL-6. All 107 exons were sequenced in DNA from seven individuals, representing 12 unique copies of *FIBL-6*. In addition to the Gln5345Arg change, only one other alteration from the NCBI reference sequence (XM_053531) was detected in the proband (III-3) from family A. This was an insertion of 11 bp between the fifth and sixth bases of exon 31. However, this homozygous insertion was also detected in five additional members of family A, 12 unrelated control subjects, and 3 additional individuals. This insertion improves a presumably unusable acceptor site to a potentially functional one (elmo.ims.u-

tokyo.ac.jp/altspl/score.html). Therefore, the insertion may better represent the genomic sequence contained in AL118512. Four synonymous (C5086T, C7600T, C13216T, and C16852G) and seven non-synonymous (Ala1624Val, Met2327Ile, Ile2418Thr, Glu2893Gly, His4084Tyr, Asp5087Val, and Arg5188His) changes were found in addition to the Gln5345Arg variation. The Ile2418Thr change, present in the 57-year old control subject, was further investigated by DHPLC. However, because this change was present in 43 of 348 chromosomes from control subjects, it appears to be a common polymorphism.

RT-PCR was used to determine if FIBL-6 is expressed in the retina (Figure 6). Two previously identified clones (AL833232 & AK027344), corresponding to the 3' end of FIBL-6, lacked exon 104, in which the Gln5345Arg variation occurs. Furthermore, intron 103 has a poor acceptor site compared to the consensus sequence for splicing (elmo.ims.u–tokyo.ac.jp/altspl/score.html). Therefore, gene-specific primers were designed that bridged or terminated in exon 104. RT-PCR analysis demonstrated FIBL-6 mRNA in human skin fibroblasts, RPE cells, retina, iris, and choroid. Alternative splicing of exon 104 occurred in FIBL-6 transcripts from all tissues examined (Figure 6).

Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced without departing from the spirit and scope of the invention. Therefore, the description should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent and patent application were specifically and individually indicated to be so incorporated by reference.